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PROGRESS REPORT ON CONTRACT
N00014-86-K0042

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CONTRACT TITLE: Energy failure of vital organs in hypovolemia and sepsis: Prevention by PGBx

START DATE: December 1, 1985

ENDING DATE: November 30, 1988

RESEARCH OBJECTIVE: The objective was to determine the efficacy of a prostaglandin B_1 oligomer 16,16-dimethyl-15-dehydro-PGB_1, $(PGB_1)_3$ in preventing cellular acute injury and energy failure after tissue ischemia or chronic sepsis. An additional goal was to characterize the mechanisms of the drug's protective effects, whenever possible.

METHODS:

Kidney ischemia. It was first necessary to determine the extent of ischemic injury that is still reversible. Renal ischemia was induced in anesthetized rats by ligating the renal artery for 30, 45, 48, or 60 minutes. After the ischemic period, reperfusion of the kidney was initiated by opening the ligature. Thirty minutes of ischemia produced inhibition of mitochondrial function that was completely reversible during the 24 hour reperfusion period. Sixty minutes of ischemia, on the other hand, produced deterioration of mitochondrial function to the extent where 24 hour reperfusion did not produce any recovery either in the presence or absence of the PGB_1 trimer. Ischemic periods extending over a 45 and 48 minute period were selected for this study. First, the effects of recovery of mitochondrial function after 45 minutes of ischemia and 24 hours of reperfusion were studied. The experimental animals were divided into two groups: those which were untreated and those to whom at the time of reperfusion 2.5 mg/kg of PGB_1 trimer was given intraperitoneally. Both untreated and treated animals were sacrificed after 24 hours of reperfusion. The kidney was removed and mitochondria isolated and their function characterized.

Isolation of kidney mitochondria. Kidney mitochondria were isolated after homogenization of the tissue in 0.3 M mannitol-sucrose-1 mM EGTA medium (MSE) at pH 7.2. A Potter-Elvehjem homogenizer was used. The homogenate was centrifuged at 700 x g for 5 minutes. The pellet was discarded and the supernatant centrifuged at 7700 x g for 14 minutes to collect the mitochondria. To wash the mitochondria, the pellet was hand-homogenized and resuspended in MSE

medium and centrifuged at 7700 x g for 7 minutes. Two additional washes were performed in 0.3 M mannitol-sucrose medium without EGTA. The final pellet was suspended in mannitol-sucrose medium at about 20-30 mg of mitochondrial protein per ml. All procedures were performed on ice (at 0-4^0 C).

Determination of mitochondrial respiratory activity. The isolated mitochondria were studied polarographically for respiratory activity with a Clark oxygen electrode in a stirred temperature controlled cuvette at 25^0 C with continuous recording. The reaction medium contained 120 mM KCl (or 0.225 M mannitol, 0.075 M sucrose), 10 mM tris-chloride and 10 mM tris-phosphate, pH 7.4. Pyruvate (6mM) and malate (2mM), ß-hydroxybutyrate (6mM), α-ketoisocaproate (6mM), and palmitylcarnitine (20μM) were used as respiratory substrates, and respiratory activities monitored in the absence and presence of added phosphate acceptor ADP (adenosine diphosphate). The rates of oxygen consumption by the mitochondria were calculated in State 3 and State 4. The respiratory control ratios were also calculated. State 3 and State 4 respiratory rates were standardized per mg of total mitochondrial protein and per mole of cytochrome oxidase.

Absorption spectra of mitochondrial respiratory chain components were determined in a Hitachi 557 dual wavelength scanning instrument. Spectra of mitochondrial samples from controls and after ischemia were studied. The concentrations of mitochondrial cytochromes aa_3, c, and b were calculated using the total change in optical density between fully oxidized and fully reduced samples and known extinction coefficients for the cytochromes: $E_{445-460}$ a(a3) = 164 mM^{-1} cm^{-1} (γ-band), $E_{560-575}$ b = 24 mM^{-1} cm^{-1}, and $E_{550-540}$ c = 20 mM^{-1} cm^{-1}. The cytochromes concentrations were expressed as nmoles cytochrome per mg of total mitochondrial protein.

Mitochondrial adenine nucleotides. The mitochondrial samples were alkalinized by adding 400 μl of 0.5 M KOH, vortexed for 15 seconds and then allowed to cool for 1 minute in an ice bath. The pH was adjusted to 6.5 - 7.0 with 180 μl 1 M KH_2PO_4 and the resulting solution was centrifuged at 10,000g for 3 minutes. The supernatant was filtered at 4^0 C using a Rainin 0.45 μm filter. The filtrate was used for nucleotide assays performed by HPLC.

Nucleotides were separated on a RESOLVE C_{18-5} column (3.9 mm X 15 cm). All samples and standards were analyzed using the following solvent gradient: 0-9 minutes, flow 0.8 ml/min with 100% 0.1 M KH_2PO_4; 9-15 minutes flow was increased linearly to 1.3 ml/min and the mobile phase was changed linearly to 2% methanol and 98% 0.1 M KH_2PO_4; 15-20 minutes, flow was maintained at 1.3 ml/min and the mobile phase was changed linearly to 10% methanol and 90% 0.1 M KH_2PO_4; 20-30 minutes, flow was maintained at 1.3 ml/min, mobile phase 10% methanol and 90% 0.1 M KH_2PO_4. Two wavelengths were employed for detection: 254 nm and 340 nm. Standard curves were constructed using external standards containing known quantities of nucleotides. Quantitation was based on peak area.

The HPLC system consisted of a Waters 600 Multisolvent Delivery System and Waters 490 Programable Multiwavelength Detector. All injections were made using a Waters 712 Wisp equipped with a sample cooling unit which was held at 4^{0}C. Data acquisition and analysis was performed using a Waters System Interface Module and an IBM-AT computer equipped with Waters 820 Chromatography software.

Pyruvate dehydrogenase. Pyruvate dehydrogenase flux in intact mitochondria, level of activation and total PDH activity were measured. The assay is based on the decarboxylation of [1-^{14}C] pyruvate and measuring the amount of liberated $^{14}CO_2$. In our modification we used 3 μl of mitochondria for incubation (approximately 30 μg of protein) per test tube containing 0.1 ml of incubation media. For determinations of active form of PDH, 3 μl of mitochondria is pipetted into test tubes with 0.2 ml of buffer solution containing same compounds as for the flux without pyruvate, ADP and hexokinase and supplemented with 6.25 mM sodium fluoride, 1 mM dithiotreitol (final concentrations). After the addition of 3 μl mitochondrial sample the samples are immediately frozen in dry ice-ethanol bath. For determination of total PDH activity the mitochondria were first incubated in the same incubation media but in the absence of fluoride and EDTA for 20 minutes then frozen in the dry ice-ethanol mixture an later assayed for PDH activity.

RESULTS.

The mitochondrial respiratory capacity was analyzed by determining the rates of oxygen utilization in state 3 during ATP synthesis and standardized as moles of oxygen utilized per mole of aa_3 per minute, using various substrates. With all substrates studied, palmityl carnitine, pyruvate, and betahydroxybutyrate respiratory activity after 45 minutes of ischemia was reduced significantly. After the 24 hour reperfusion period, respiratory activities with palmityl carnitine as substrate recovered in the absence or presence of PGB_1 trimer to practically normal level. The respiratory activities with pyruvate, however, declined further during the reperfusion period in the untreated animals but recovered to a certain extent in the treated animals. Betahydroxybutyrate supported respiration remained unchanged during the reperfusion period in the untreated animals but recovered close to normal level in the treated animals. (See Figure 1)

Figure 2 illustrates changes in cytochrome concentrations after ischemia and reperfusion. Cytochrome c declined significantly below control level after 45 minutes of ischemia. Some recovery occurred during the 24 hour reperfusion in the untreated animals and further recovery in the treated animals. Cytochrome oxidase similarly declined below normal during the ischemic period and some recovery occurred in the untreated and further recovery in the treated animals. There were no significant changes in cytochrome b concentrations in these experimental groups.

In the next series of experiments, the ischemic period was extended to 48 minutes. In the untreated animals, 24 hour reperfusion period did not provide any recovery of mitochondrial respiratory capacity in state 3 (Figure 3 and Table I). The respiratory capacity measured as moles oxygen utilized per mole aa_3 per minute with various substrates declined beyond the ischemic lever with palmityl carnitine, pyruvate, and betahydroxybutyrate as substrates. In the treated animals, however, the substrate oxidation with all three substrates recovered to a large extent and reached normal level with palmityl carnitine as the substrate. Cytochrome aa_3 and cytochrome c did not recover during the reperfusion in the untreated animals (Figure 4). However, in the treated animals both cytochromes aa_3 and c reached essentially normal values after the 24 hour reperfusion. Mitochondrial calcium transport activities were also determined

in these animals. Calcium transport activity after 48 minutes of ischemia declined practically to 0 uptake. After the 24 hour reperfusion period some recovery of the calcium transport activity occurred in the untreated animals and a complete recovery occurred in the treated animals (Figure 5). Our data thus indicate that a bolus injection of PGB_1 trimer given at the time of reperfusion provides significant improvement of the mitochondrial function after 48 minutes of renal ischemia.

Utilization of different fuels seem to be differentially affected by ischemia. It appears that pyruvate oxidation is more sensitive than oxidation of palmityl carnitine as fuel. For that reason, we also determined pyruvate dehydrogenase activity changes in kidney mitochondria after the ischemic period as well as after reperfusion. The data are presented in Table II. Ischemia, with or without reperfusion, does not alter the level of total PDH activity significantly. The flux through the active enzyme, however, is significantly decreased after ischemia, but it recovers to normal level after 24 hours of reperfusion with or without $(PGB_1)_3$ treatment.

To determine the possible mechanisms of ischemic cell injury at the mitochondrial level, we studied some parameters relating to oxygen free radical responses. Glutathione peroxidase, glutathione reductase activities, total glutathione levels and manganese SOD activities were determined in normal controls, sham controls and ischemic reperfused animals with and without treatment. As shown in Table III none of these parameters showed any significant differences after 24 hour reperfusion in the untreated or treated groups as compared to sham controls. It thus appears that the mitochondrial functional deterioration is not related to any of these measured free radical enzyme responses.

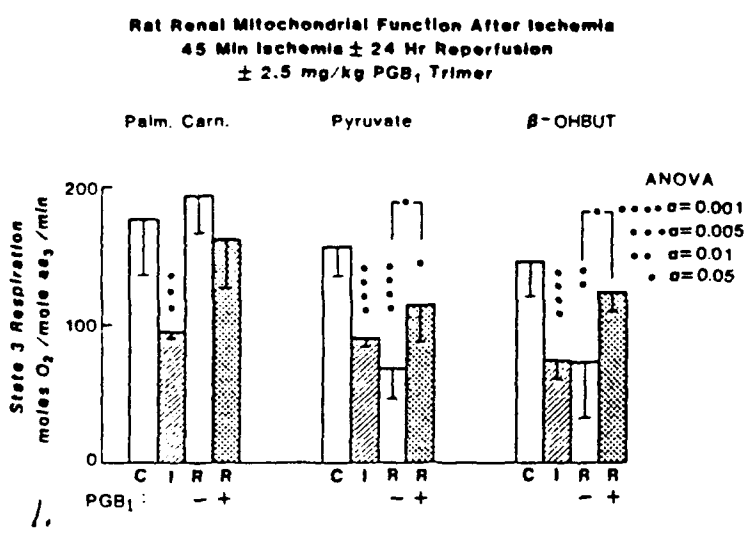
In the next set of experiments we encountered some difficulties. It appears that the rats used for these experiments were much more resistant to ischemia. The previous experiments had been preformed during the winter and spring seasons. The new series was initiated during the summer and continued into the fall. It is possible that seasonal variations in rats were responsible for the differences. After many attempts at various lengths of ischemia (from 50-60 minutes) we finally decided to complete the study in spite of the fact that 24 hours of reperfusion resulted in nearly complete recovery of the mitochondrial parameters without $(PGB_1)_3$ treatment. $(PGB_1)_3$ treatment did not result in any improvement of the mitochondrial functional parameters (see Table IV). In 16 of these animals we also determined the mitochondrial adeninenucleotides and hypoxanthine (HX) and xanthine (XAN) concentrations by HPLC techniques. Figure 6 is a typical chromatogram of a mitochondrial sample showing the separation of he components to be measured and Table V summarizes the data. The sum of ATP + ADP + AMP is highly reduced in the mitochondrial samples from the ischemic samples, and HX and XAN are measurable in these samples. One hour of reperfusion results in the disappearance of the HX and XAN but no recovery of the sum of adenine nucleotides. A partial recovery of the adenine nucleotides is achieved after 24 hours of reperfusion in the absence or presence of $(PGB_1)_3$ treatment.

The variability in the resistance of rats to ischemic injury is an unfortunate turn of events, and it casts some serious questions on the validity of the $(PGB_1)_3$ treatment data. Unfortunately, when studies were initiated with PGB_1 - dimer, similar experimental problems prevailed. No differences between treated

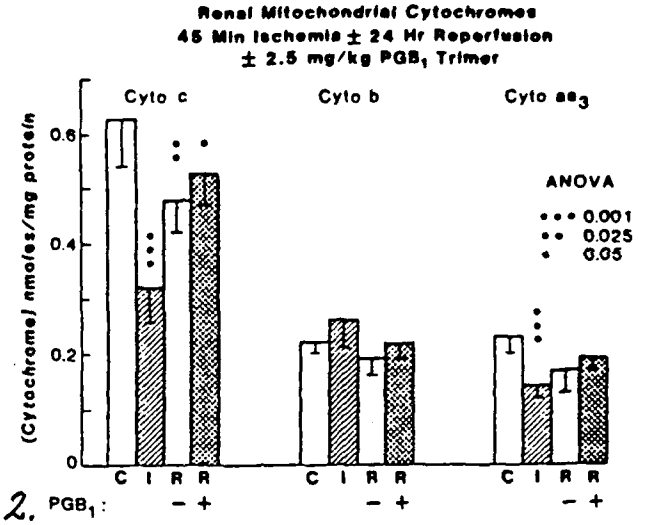
and untreated animals were found in any mitochondrial parameters studied.

CONCLUSION.

On the basis of our data we conclude that $(PGB_1)_3$ can be effective in preventing cellular reperfusion injury, as quantitated by measurements of mitochondrial function, but only under some narrowly defined conditions. It appears that these conditions are so limited that solid experimental proof of the drug's efficacy is difficult to generate.

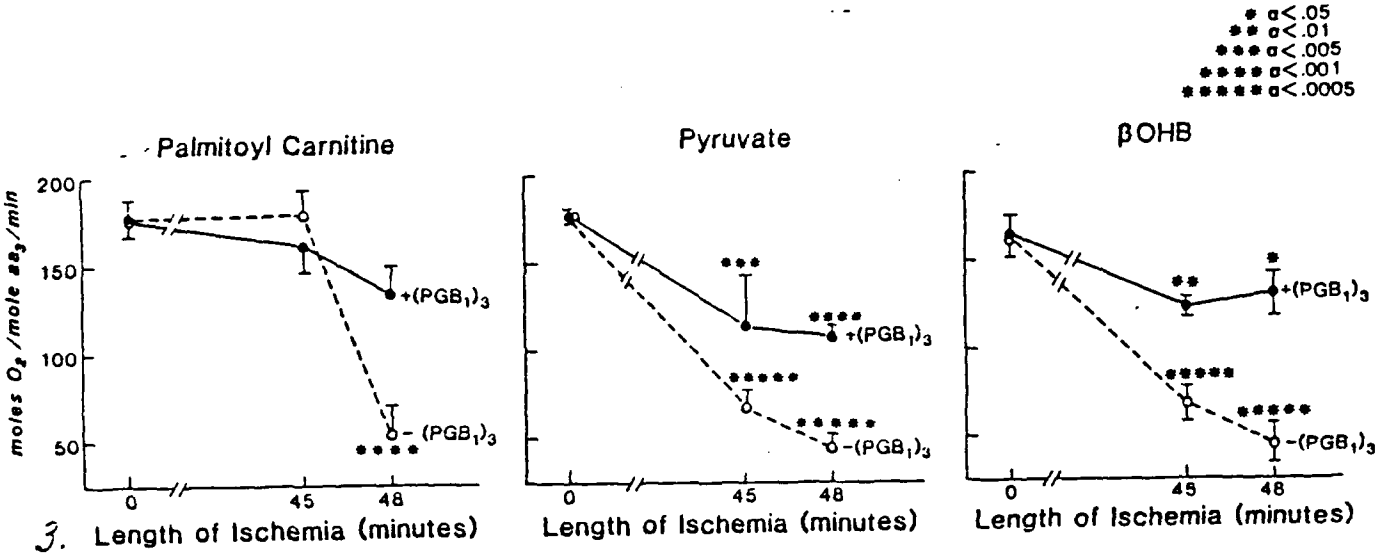


1.



2.

State 3 Respiratory Activity Using Various Substrates After Ischemia and 24 hours of Reperfusion



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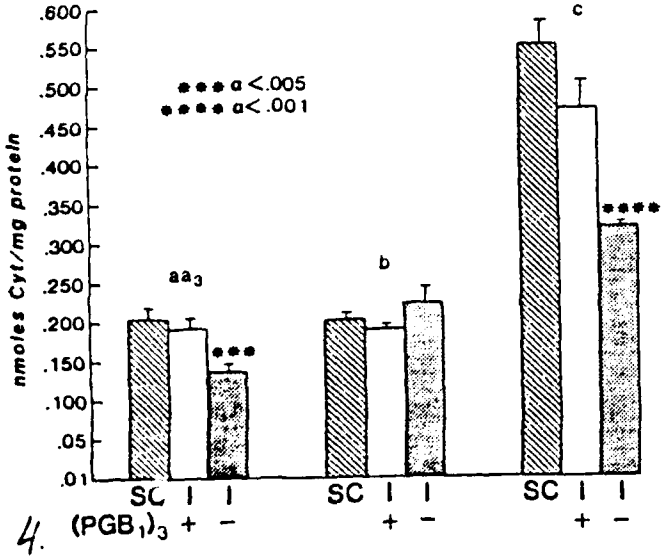

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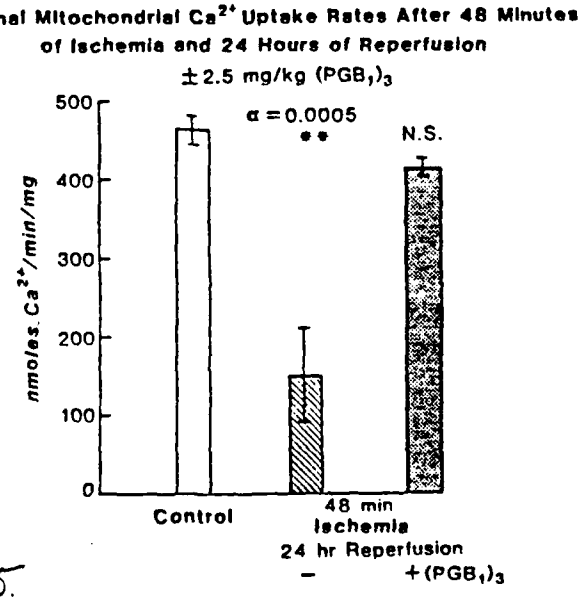
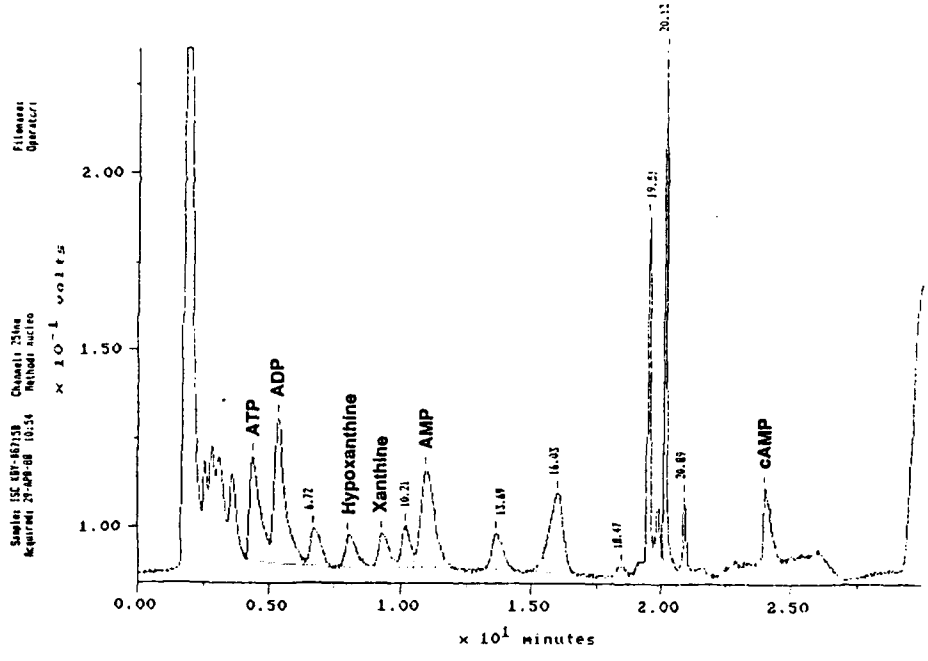


5.

60 min Ischemia, no Reperfusion
Protein 0.101 mg/Sample



55 min Ischemia, 24 hrs Reperfusion
Protein 0.094 mg/Sample

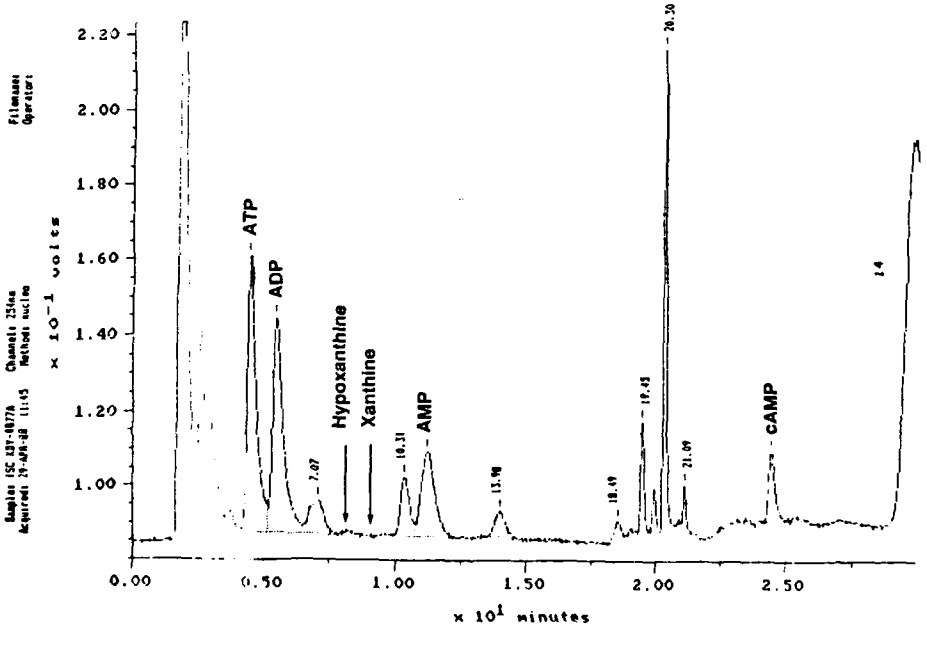


TABLE I

	State 3 Rate moles O_2/mole aa_3/min			[Cytochrome] nmoles/mg Protein			Ca^{2+} uptake nmoles/min/mg
	PC	PYR	βOHB	aa_3	b	c	
Control	177±13	177±9	167±9	.21±.01	.20±.008	.55±.03	437±1
45 min Isch	95±4	87±5	74±19	.13±.02	.23±.002	.30±.08	0
48 min Isch +24 hrs Reperf.							
-$(PGB_1)_3$	59±19	45±9	45±13	.14±.01	.23±.02	.30±.001	150±138
+$(PGB_1)_3$	162±15	114±29	122±5	.19±.01	.22±.01	.53±.02	413±23

Our data indicate that a bolus injection of $(PGB_1)_3$ given at the time of reflow provides significant improvement of mitochondrial function after 48 minutes of renal ischemia in the rat.

TABLE II

PDH ACTIVITIES OF KIDNEY MITOCHONDRIA AFTER ISCHEMIA

	nmoles/min/mg FLUX	TOTAL ACTIVITY
SHAM CONTROL n=6	7.3 ± 2.1	54 ± 10
45 MIN ISCHEMIA n=6	4.7 ± 1.2 * (p<0.025)	49 ± 20
ISCHEMIA + 24 HR REPERFUSION		
-$(PGB_1)_3$ n=9	8.2 ± 2.7	49 ± 17
+$(PGB_1)_3$, 2.5mg n=3	7 ± 3	40 ± 12
+$(PGB_1)_3$, 1mg n=3	8.5 ± 3.7	48 ± 18

TABLE III

ISCHEMIC RAT KIDNEY MITOCHONDRIA

48 MIN ISCHEMIA

24 HR REPERFUSION

	GLUTATHIONE PEROXIDASE	GLUTATHIONE REDUCTASE	TOTAL GLUTATHIONE	Mn SOD
	n moles NAD(P)H oxid. / min x mg	n moles NAD(P)H oxid. / min x mg	n moles/mg	UNITS/mg
NORMAL CONTROL n = 3	32 ± 5.9	29 ± 3.0	0.21 ± 0.03	21 ± 4.9
SHAM CONTROL n = 5	26 ± 10.7	32 ± 6.2	0.24 ± 0.02	21 (1)
ISCHEMIC - REPERFUSED				
$-(PGB_1)_3$ n = 15	25 ± 2.6	32 ± 5.9	0.21 ± 0.04	18 ± 1.8 (2)
$+(PGB_1)_3$ n = 17	23 ± 6.3	33 ± 6.1	0.20 ± 0.04	16 ± 5.4 (4)

TABLE IV

KIDNEY MITOCHONDRIAL FUNCTION AFTER ISCHEMIA

	STATE 3 RATE moles O_2/min/mole aa_3			CYTOCHROME nmoles/mg protein	
	PC	PYR	BOHB	aa_3	c
SHAM CONTROL n=5	188 ± 12	139 ± 16	132 ± 9	0.23 ± 0.03	0.50 ± 0.07
55 MIN ISCHEMIA n=4	60 ± 9 ***	68 ± 19 ***	60 ± 6 ***	0.15 ± 0.03 **	0.23 ± 0.05 ***
55 MIN ISCHEMIA + 24 HR REPERFUSION					
$-(PGB_1)_3$ n=8	177 ± 33	126 ± 22	143 ± 25	0.18 ± 0.03 *	0.43 ± 0.10
$+(PGB_1)_3$ n=4	153 ± 27	121 ± 13	109 ± 28	0.18 ± .03	0.51 ± .05

*** $p<0.001$
** $p<0.005$
* $p<0.01$

TABLE V

CONCENTRATIONS OF KIDNEY MITOCHONDRIAL ADENINE NUCLEOTIDES, XANTHINE AND HYPOXANTHINE

nmoles/mg protein

	ATP + ADP + AMP	HX	XAN
SHAM CONTROL n=4	7.0 ± 1.0	0	0
ISCHEMIA 55 MIN n=4	3.6 ± 0.6 * * P<0.0025	0.46 ± .21	0.46 ± .16
ISCHEMIA + 1 HR REPERFUSION n=4	3.4 ± 0.4 * * p<0.0025	0	0
ISCHEMIA + 24 HR REPERFUSION n=4	5.6 ± 0.3 * p<0.05	0	0
-$(PGB_1)_3$ n=2	5.65 ± 0.5		
+$(PGB_1)_3$ n=2	5.65 ± 0.07		

Volume 21, Number 4, 1987

CIRCULATORY SHOCK

THE SHOCK SOCIETY PRESENTS
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MONTREAL, CANADA
JUNE 7-11, 1987

188 TRIMER OF 15-DEHYDRO-PGB_1 IMPROVES RECOVERY OF MITOCHONDRIAL FUNCTION AFTER RENAL ISCHEMIA. Linda L. Widener, Dagmar Bartos and Leena Mela-Riker, Departments of Surgery, Biochemistry and Pediatrics, Oregon Health Sci. Univ., Portland, OR.

Oligomeric mixtures of prostaglandin B_1 are protective against mitochondrial functional failure after tissue ischemia. The active component of the PGB_1 mixture is unknown. We used the trimer of 15-dehydro-PGB_1 to test its protective effect in renal ischemia. Renal ischemia was induced by a unilateral closure of the renal artery in the rat. After a 45 min. ischemic period the arterial clip was opened to recover renal blood flow. At this time the treated animals received a bolus of 2.5 mg/kg 15-dehydro-PGB_1, IP. The untreated animals received an injection of the vehicle. The animals were sacrificed after 24 hours, renal mitochondria were isolated and their function analyzed. The data are shown in the Table.

*p < 0.05

	[CYTOCHROME] n moles/mg			STATE 3 RATE moles O_2/mole aa_3/min	
	aa_3	b	c	pyruvate	β-OH butyrate
CONTROL	0.24±.03	0.22±.03	0.63±.1	158±23	146±28
ISCH 45 MIN	0.13±.02*	0.23±.002	0.30±.08*	87±5*	74±19*
24 - PGB_1	0.17±.04*	0.19±.03	0.47±.06*	63±31*	76±41*
HRS + PGB_1	0.19±.01	0.22±.03	0.51±.05	102±28*	115±30

These data indicate that a bolus injection of the trimer of 15-dehydro-PGB_1 given at the time of reflow provides significant improvement of mitochondrial function after 45 min of renal ischemia in the rat. Supported by Office of Naval Research.